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TECHNICAL MANUSCRIPT 351

# APPLICATION OF THE SINGLE-DOSE ASSAY TECHNIQUE TO PSITTACOSIS

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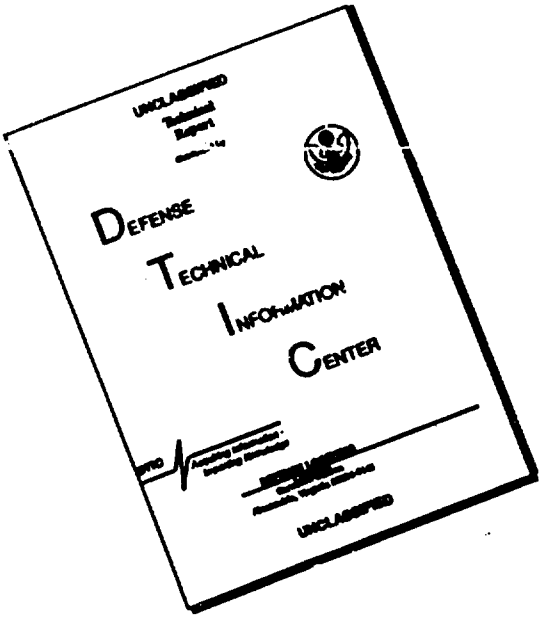


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# APPLICATION OF THE SINGLE-DOSE ASSAY TECHNIQUE TO PSITTACOSIS

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Product Development Division
AGENT DEVELOPMENT AND ENGINEERING LABORATORY

Project 1B533601D426 January 1967

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

## ABSTRACT

When suspensions of psittacosis organisms were injected intracerebrally into groups of mice, a nearly linear relationship was observed between the concentration of the agent injected and the mean time to death of the mice. Thirty-four psittacosis preparations were assayed and by plotting the relationship between the reciprocal time to death for mice given the $10^{-3}$ dilution of agent and the $MICLD_{50}$ values for the preparations, a reference curve was established. This reference curve made it possible to estimate directly the $LD_{50}$ value of a psittacosis suspension of unknown concentration from the mean reciprocal time to death of a group of mice injected with a single dilution. In this work, the number of mice used was reduced by 62.5%, the titrations were complete in 3 to 5 days compared with the usual 12 days, three to four times as many assays could be done in a day, and no assays had to be repeated because end points were not missed. In addition, the precision of the single-dilution assay compared favorably with that of the $LD_{50}$ titration.

# I. INTRODUCTION

Golub in 1948[1] described a single-dilution method for estimating $LD_{50}$ titers. This procedure has become one of the most commonly used methods for titrating the psittacosis-LGV group of agents. This single-dilution assay is based on the linear inverse relationship between the amount of agent injected and the mean time to death (MTD) of the eggs. From this relationship it is possible to estimate the titer based on the MTD of a group of eggs injected with a single dilution.

The single-dose method of estimating $LD_{50}$ values for the psittacosis group has been used successfully in studies on: (i) the growth cycle in eggs,[2] (ii) the development of antibiotic-resistant strains,[3,4] and (iii) the propagation of psittacosis in tissue culture.[5,6] These investigators reported these results as egg $LD_{50}$ values. However, we were interested in using the mouse as a host because of the great seasonal variation in the quality of our eggs, and also because of the loss of animal infectivity with no corresponding loss in egg infectivity following treatment with certain solvents.[7] A dose correlation in the titration end points in embryonated eggs and in mice was reported for psittacosis[1] and for variola virus,[8] so it seemed feasible to establish a single-dilution assay in mice. Establishing such an assay, by which we could estimate the $LD_{50}$ value, would result in considerable savings in time and in cost of mice.

The purpose of this report is to show (i) that there is a relationship between dose and time to death for psittacosis in mice, (ii) that it is reliable to estimate directly the $LD_{50}$ value of psittacosis materials from the reciprocal MTD of a group of mice injected with a single dilution, and (iii) that the precision of the single-dilution assay compares favorably with that of the $LD_{50}$ titration.

# II. MATERIALS AND METHODS

## A. AGENT

The Borg strain of the psittacosis group was used in this study. A $10^{-2}$ suspension of infected yolk sac material was prepared in heart infusion broth (Difco). This suspension was blended for one minute in a Waring Blendor (semimicro size) and further 10-fold dilutions were made in heart infusion broth.

## B. ASSAY PROCEDURE

Groups of 10 mice, Swiss-Webster strain, weighing 10 to 14 grams were inoculated intracerebrally with 0.03 ml of the diluted agent. To obtain initial data on dose and time to death, all dilutions from $10^{-2}$ to $10^{-8}$ were used. From these data, the $10^{-3}$ dilution was selected for the single-dose assay, and the $10^{-5}$ to $10^{-8}$ dilutions were used to bracket the 50% end point.

Mice were observed for 12 days and deaths were recorded at 8:00 AM and 2:00 PM daily. Deaths prior to 24 hours after inoculation were assumed to be due to inoculation trauma. Deaths discovered at successive observation periods were assumed to have occurred at random in the interval between the periods, and the midpoint of the interval was used as the best estimate of time of death. Thus, a mouse alive at one observation period and dead at the following was considered to have died at the hour halfway between the two periods. Mice alive and well at the end of 12 days were considered to be uninfected.

The reciprocal transformation of Brownlee and Hamre[9] was used in calculating MTD values. The reciprocal of the time to death was multiplied by 100 to provide whole numbers for ease in computation.

Groups of eight 7-day-old embryonated eggs were inoculated via the yolk sac with 0.25 ml of appropriate dilutions prepared for the assays in mice. The eggs were candled daily at 8:00 AM and deaths were recorded. Embryos dying prior to 48 hours were regarded as deaths from nonspecific causes; embryos surviving 10 days after inoculation were considered to be uninfected.

The $LD_{50}$ value for both eggs and mice was calculated by the method of Reed and Muench.[10]

## C. STATISTICAL ANALYSIS

All statements indicating significant differences due to host and assay method are based on analyses of variance and F tests at the 5% level of probability.

## III. RESULTS

Preliminary titrations of suspensions of psittacosis were made to gain initial information on dose and time to death relationship. As shown in Figure 1, a linear dose-time relationship was found in the range of concentrations from $10^{-4}$ to $10^{-9}$ in eggs and from $10^{-2}$ to $10^{-8}$ in mice. The slope of the line is steeper for mice than for eggs, which may indicate a more rapid growth of the agent in the mouse tissue than in egg tissue. These linear responses indicated that both $LD_{50}$ titration and single-dose assays were feasible in both embryonated eggs and mice.

The $LD_{50}$ values for 20 preparations titrated in both eggs and mice are given in Table 1. There was no significant difference between the values obtained in the two hosts. Also, the inherent variability within each host was not significantly different.

From the dose response relationship shown in Figure 1, the $10^{-3}$ dilution was selected for the single-dose assay in mice. An MTD of about 63 hours was obtained with this dilution, which (i) assured definition between deaths from trauma and those from infection and yet assured 100% mortality, and (ii) limited observation of mice to twice daily.

Thirty-four psittacosis preparations were assayed for MTD and $LD_{50}$ values. The $\log_{10}$ mouse intracerebral $LD_{50}$ ($MICLD_{50}$) titers ranged from 4.0 to 9.0. The materials were grouped by titer by half-log intervals and the relationship between the reciprocal of the MTD for the $10^{-3}$ dilution and the $LD_{50}$ value is shown in Figure 2. This is the reference curve that was used in all further assays to estimate the $LD_{50}$ value from the MTD of groups of mice injected with the $10^{-3}$ dilution.

An additional 150 preparations were assayed in triplicate, using the MTD value to estimate the $MICLD_{50}$ titer. In about 10% of these assays, selected at random, a standard $LD_{50}$ titration also was done. The estimated $LD_{50}$ values from the single-dilution assay and the values calculated according to Reed and Muench are shown in Table 2. There was no significant difference between the estimated and calculated values, and the inherent variability within each method was not significantly different.

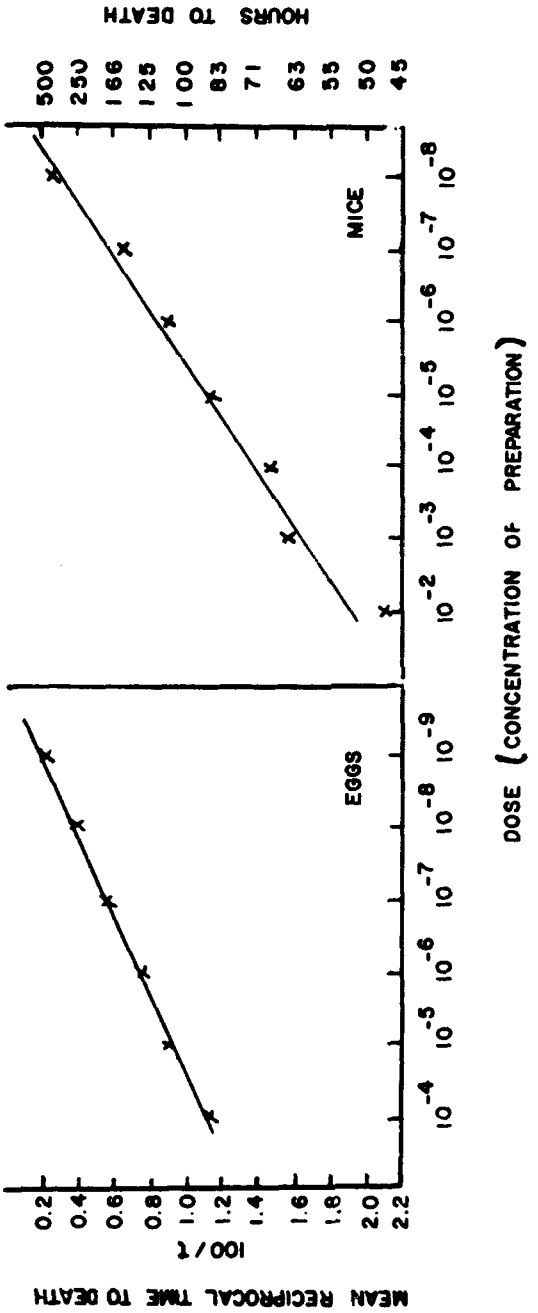


Figure 1. Relationship of Dose to Mean Time to Death for Eggs and Mice Given Suspensions of Psittacosis Virus.

TABLE 1. $LD_{50}$ VALUES OBTAINED FROM TWO HOSTS GIVEN PSITTACOSIS

| Preparation | Host: Embryonated Eggs, titer | Host: Mice, titer |
|---|---|---|
| 1 | 7.1 | 5.7 |
| 2 | 6.5 | 5.2 |
| 3 | 6.5 | 5.7 |
| 4 | 5.3 | 5.0 |
| 5 | 5.9 | 6.0 |
| 6 | 6.0 | 5.4 |
| 7 | 7.6 | 7.7 |
| 8 | 7.0 | 7.2 |
| 9 | 8.7 | 7.3 |
| 10 | 7.2 | 7.9 |
| 11 | 5.3 | 5.5 |
| 12 | 9.1 | 8.6 |
| 13 | 6.4 | 7.3 |
| 14 | 7.5 | 7.8 |
| 15 | 7.8 | 7.6 |
| 16 | 7.3 | 7.4 |
| 17 | 6.6 | 6.5 |
| 18 | 7.1 | 6.3 |
| 19 | 8.4 | 8.4 |
| 20 | 8.6 | 8.3 |
| $\bar{X}$ | 7.1 | 6.8 |

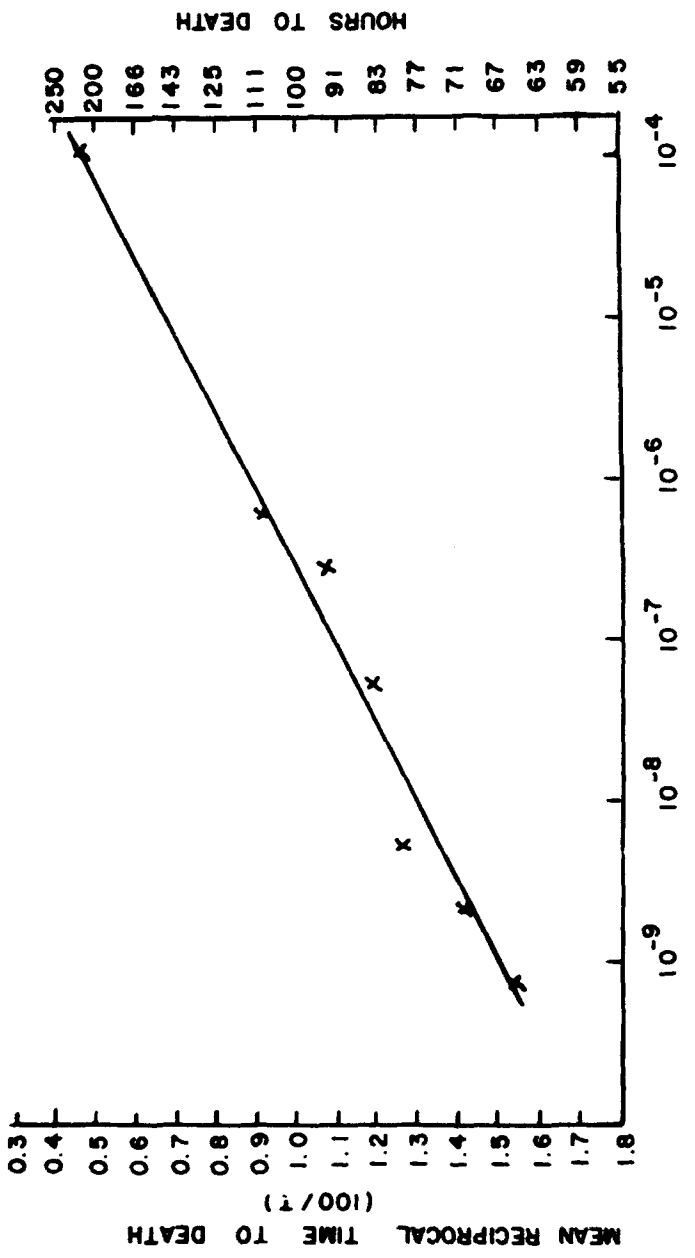


Figure 2. Reference Curve for Estimating $MICLD_{50}$ Titer of Psittacosis Virus from Mean Reciprocal Time to Death for Groups of Mice Injected with the $10^{-3}$ Dilution.

TABLE 2. $LD_{50}$ VALUES OF PSITTACOSIS PREPARATIONS OBTAINED BY TWO METHODS

| Preparation | Estimated from Reference Curve[a], titer | Calculated by Reed Muench Method, titer |
|---|---|---|
| 1 | 6.8 | 7.8 |
| 2 | 6.8 | 7.5 |
| 3 | 6.6 | 8.2 |
| 4 | 6.5 | 8.0 |
| 5 | 7.8 | 7.8 |
| 6 | 7.1 | 8.0 |
| 7 | 7.0 | 7.8 |
| 8 | 8.1 | 8.1 |
| 9 | 7.4 | 8.6 |
| 10 | 8.1 | 8.3 |
| 11 | 5.7 | 5.5 |
| 12 | 5.9 | 5.5 |
| 13 | 5.8 | 5.5 |
| 14 | 5.5 | 5.5 |
| 15 | 8.1 | 8.6 |
| 16 | 7.3 | 8.2 |
| 17 | 7.6 | 8.1 |
| 18 | 8.6 | 8.9 |
| 19 | 7.6 | 8.2 |
| 20 | 9.1 | 8.1 |
| 21 | 6.4 | 7.2 |
| 22 | 6.3 | 7.4 |
| 23 | 6.8 | 7.0 |
| 24 | 7.1 | 7.9 |
| 25 | 7.5 | 7.3 |
| 26 | 7.0 | 7.6 |
| 27 | 8.1 | 8.0 |
| 28 | 7.4 | 7.8 |
| 29 | 7.9 | 7.9 |
| 30 | 7.8 | 7.3 |
| 31 | 8.1 | 8.8 |
| 32 | 8.0 | 8.2 |
| 33 | 6.8 | 8.0 |
| 34 | 7.4 | 7.8 |
| 35 | 8.0 | 9.3 |
| 36 | 9.0 | 9.0 |
| 37 | 6.9 | 6.5 |
| 38 | 7.9 | 7.1 |
| 39 | 8.4 | 9.5 |
| 40 | 8.3 | 8.9 |
| 41 | 6.9 | 8.2 |
| 42 | 7.2 | 7.8 |
| 43 | 7.8 | 8.4 |
| 44 | 8.7 | 8.6 |
| $\bar{X}$ | 7.4 | 7.8 |

a. See Figure 2.

## IV. DISCUSSION

Since Golub[1] established a single-dilution assay for the psittacosis group of viruses, several variations of his method have been introduced. Crocker and Bennett[11] adopted a slope assay that substituted three or more serial lethal viral dilutions for the single lethal dilution used by Golub; it had no relationship to any estimate of $LD_{50}$ value. They assert that greater precision was obtained with their slope assay than with a single-dilution assay. However, Smith and Westgarth,[12] in attempting to use the slope assay in their work on several neurotropic viruses, found no advantage was achieved. Bauer,[13] working with neurovaccinia, ectromelia, dengue I, rabies, and yellow fever viruses, correlated a single-dilution assay to zero mortality ($D_0$) units that bore a fixed relationship to the $LD_{50}$ value. Admittedly, the single-dilution titration in $D_0$ units has advantages over the usual $LD_{50}$ titration, but as Bauer then related the $D_0$ unit to an $LD_{50}$ value, there seems to be no advantage over Golub's direct correlation of single-dilution titration to $LD_{50}$ value.

Dougherty et al.[14] include an $LD_{50}$ titration on a standard virus suspension when each group of unknown virus preparations is assayed by the single-dilution method. This is essentially the method we have found satisfactory except that the choice of preparations that are titered by both single-dilution assay and by $LD_{50}$ method is a random selection. A control preparation is always titered with each group of unknown preparations, but the $LD_{50}$ titration may be made either on the control or on an unknown preparation.

Regardless of the variations in Golub's original method, all of the investigators quoted are firm in their belief that the graded response represented by a measurement of the relationship of dose and time to death has many advantages over a quantal response such as the $LD_{50}$ titration. The most frequently discussed advantage of such an assay is using one or even three dilutions over the conventional method of using four to six dilutions and attempting to bracket the 50% end point. Other advantages are that (i) fewer hosts are needed, (ii) the titration is completed in a shorter time, (iii) the time necessary for preparing dilutions and inoculating the hosts is minimized, and (iv) with materials of unknown concentration, the end point is not missed by an inaccurate choice of dilutions. In addition, using a reciprocal transformation allows each host to contribute a numerical estimate of the amount of agent (virus, bacteria, fungus, or toxin) inoculated into it, and the variance of responses is stabilized over the whole range of doses.

In our work with psittacosis virus, the number of mice used was reduced by 62.5%, the titrations were complete in 3 to 5 days compared with the usual 12 days, three to four times as many assays could be done in a day, and no assays had to be repeated because we did not miss end points. In addition, the precision of the single-dilution assay compared favorably with that of the $LD_{50}$ titrations.

As we found in establishing a similar single-dilution assay for Venezuelan equine encephalomyelitis virus,[15] a uniform supply of hosts is imperative. This is particularly true with mice that become more resistant with age to many infections. A reference curve established for mice averaging 12 grams cannot be used for those that weigh 18 grams. The heavier mice live 24 hours longer, give smaller mean reciprocal values, and give $LD_{50}$ values that may be as much as a log lower than when 12-gram mice are used. Also, changing from one strain of mouse to another would necessitate establishing a new reference curve, as does changing from one flock to another for embryonated eggs.[14]

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13 ABSTRACT

When suspensions of psittacosis organisms were injected intracerebrally into groups of mice, a nearly linear relationship was observed between the concentration of the agent injected and the mean time to death of the mice. Thirty-four psittacosis preparations were assayed and by plotting the relationship between the reciprocal time to death for mice given the $10^{-3}$ dilution of agent and the $MICLD_{50}$ values for the preparations, a reference curve was established. This reference curve made it possible to estimate directly the $LD_{50}$ value of a psittacosis suspension of unknown concentration from the mean reciprocal time to death of a group of mice injected with a single dilution. In this work, the number of mice used was reduced by 62.5%, the titrations were complete in 3 to 5 days compared with the usual 12 days, three to four times as many assays could be done in a day, and no assays had to be repeated because end points were not missed. In addition, the precision of the single-dilution assay compared favorably with that of the $LD_{50}$ titration.

14. Key Words

| | |
|---|---|
| *Psittacosis | Lethal |
| *Assay | Mice |
| *Dose | Embryonated eggs |
| Death | Titer |

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